GENERATION OF *ARABIDOPSIS THALIANA* LINES WITH SUPPRESSED *ATNDA1* UTILIZING RNA INTERFERENCE

(Sintasan Generasi *Arabidopsis thaliana* dengan Atnda1 Tersupresi Memanfaatkan Interferensi RNA)

Wardiah
Program Studi Biologi, FKIP, Universitas Syiah Kuala
Email: wardiah.fkip@gmail.com

Abstract
Atnda1 is one of matrix NAD(P)H dehydrogenase genes in *Arabidopsis thaliana*. Its function is still unclear. This is an encouragement to study the gene by disrupting the expression of the gene. This study was aimed to generate Arabidopsis thaliana possessing suppressed *Atnda1* utilizing RNA interference. Double stranded *nda1*-RNAi silencing was chosen for this project. The generation of the lines was started by creating specific primer for *Atnda1*. The creation of *nda1-RNAi* cassette used the Gateway cloning. We utilized *Agrobacterium* strain GV3101 as a mediator to transfer the cassette into the plants. Dipping the flower into Agrobactrium solution was done to transfer the *nda1-RNAi* cassette into wild type *Arabidopsis*. The seeds collected from the mutant plants were checked in selective media. Contamination was the daunting problem in the process of checking the plants. This was crucial to ensure that the insertion has been done successfully. However, selection of the mutants must be done before analysis of the function is carried on the mutants.
Key words: *Matrix NAD(P)H Dehydrogenase Genes, Atnda1, Arabidopsis thaliana, RNA interference*

Abstrak
Atnda1 adalah salah satu matriks (P) gen dehidrogenase NAD H di *Arabidopsis thaliana*. Fungsinya masih belum jelas. Ini merupakan dorongan untuk mempelajari gen dengan memdodifikasi ekspresi gen. Penelitian ini bertujuan untuk menghasilkan *Arabidopsis thaliana* yang memiliki Atnda1 tersupresi menggunakan interferensi RNA. nda1-RNAi utas ganda digunakan dalam penelitian ini. Generasi garis dimulai dengan menciptakan primer spesifik untuk Atnda1. Penciptaan kaset nda1-RNAi menggunakan kloning Gateway. Kami menggunakan galur Agrobacterium GV3101 sebagai mediator untuk mentransfer kaset ke dalam tanaman. Dipping bunga ke dalam larutan *Agrobactrium* dilakukan untuk mentransfer kaset nda1-RNAi ke *Arabidopsis* tipe liar. Benih dikumpulkan dari tanaman mutan diperiksa dalam media selektif. Kontaminasi adalah masalah utama dalam proses memeriksa tanaman. Penyisipan telah dilakukan dengan sukses penting untuk diperhatikan. Namun, pemilihan mutan harus dilakukan sebelum analisis fungsi ini dilakukan pada mutan tersebut.
Kata Kunci: *Matrix NAD(P)H Dehydrogenase Genes, Atnda1, Arabidopsis thaliana, interferensi RNA*

INTRODUCTION

Respiration occurs in a cell and is associated with energy production accumulated in a compound called ATP. Cells utilise ATP for maintenance and development.Aerobic respiration is common to almost eukaryotic organisms including plants. There are three main processes that occur in different places in the cell; glycolysis is in cytosol and citric acid cycle and oxidative phosphorylation take place in the matrix and inner membrane of mitochondria (Taiz and Zeiger, 2002).

The three main processes play important role of their own. Glycolysis and the citric acid cycle generate ATP or NADH and FADH2. The oxidation of NADH and FADH2 generates electrons that are passed along the electron transport chain in the inner membrane of mitochondria to reduce oxygen and form water. Electrons transported along the chain drives energy formation as ATP by oxidising ADP (Karp, 1996).

The series processes of electrons transport along the inner membrane of mitochondria have involved protein-lipid enzymecomplexes which play certain roles. These complexes are NADH ubiquinonereductase (Complex I) which oxidizes NADH producing up to three ATPs, succinate-ubiquinone reductase (Complex II) which functions to transfer electrons initially produced in succinate oxidation to ubiquinone (Coffee, 1999), Complex III which reduces ubiquinone and ubiquinol and transfers the electrons to cytochrome c, and Cytochrome c oxidase (Complex IV) that accepts electrons from cytochrome c and donates those to oxygen.

Unlike animal mitochondria, plants have three unique pathways in the process of electron transport known as alternative respiratory pathways. The first pathway is branched to bypass the Complex III pathway in the presence of cyanide, an inhibitor of Complex III (Vanlerberghe and McIntosh, 1997). Another two pathways known as alternative dehydrogenases are present which bypass proton-pumping Complex I and transfer electrons from NADH to ubiquinone without proton translocation (Svensson and Rasmusson, 2001).

The alternative NAD(P)H dehydrogenases consist of NADH dehydrogenases located on the outer surface of the mitochondrial inner membrane catalyzing oxidation of cytosolic NADH and NADH

called external NAD(P)H dehydrogenases, and matrix NADH and NADPH dehydrogenases located in the inner surface of the inner membrane of mitochondria called internal NAD(P)H dehydrogenases (Finnegan *et al*., 2004). In yeast (*Saccharomyces cerevisiae*), there are several alternative NAD(P)H dehydrogenases found in its respiratory chain; *ScNDE1*, *ScNDE2* (external facing), and *ScNDI1* (internal facing) (Moore *et al*., 2003). In potatoes, two NDH genes were found; *Stnda1* and *Stndb1*, encoding internal and external enzymes respectively (Rasmusson and Agius, 2001). In *Arabidopsis*, seven genes which encode putative type II have been identified (Michalecka *et al.*, 2004).

The seven putative genes encoding type II NAD(P) H dehydrogenases are categorized into three families; *Atnda*, *Atndb* and *Atndc* (Michalecka *et al*., 2004).The *Atnda* has two putative open reading frames (ORFs); *Atnda1* and *Atnda2*, while *Atndb* posses four different ORFs; *Atndb1, Atndb2, Atndb3* and *Atndb4.* The *Atndc1* only has ORF which is *Atndc1*. Sequence analysis revealed that *Atnda* and *Atndb* gene families are highly analogous to NDI and NDE in yeast and potato (Michalecka *et al*., 2003). Intra-mitochondrial localisation studies propose that *nda1, nda2* and *ndc1* are located in the inner surface of the inner mitochondrial membrane and while the *ndb* family genes attach to the outer surface of the inner membrane of mitochondria (Elhafez *et al*., 2006; Michalecka *et al*., 2003). Previously, Green fluorescence protein (GFP) analysis confirmed that *nda1, nda2, ndc1, ndb1,* and *ndb2* target mitochondria (Michalecka *et al*., 2003). However, a more recent study found that four type II NAD(P)H dehydrogenases in *Arabidopsis* target two locations in cell; *ndc1* target to mitochondria and chloroplasts whereas *nda1*, *nda2* and *ndb1* target mitochondria and peroxisomes (Carrie *et al*., 2008).

The presence of internal and external dehydrogenases together with alternative oxidases plays an important role in maintaining redox balance, thermogenesis, cold resistance, and photosynthetic metabolism (Vanlerberghe and McIntosh, 1997). Alternative dehydrogenases has involved during thermogenic respiratory burst during floral development.

Most organisms have a natural mechanism to protect their genome against viral infection and the intervention transposable elements or repetitive sequences. The mechanism involves dsRNA that induces the interruption of the target gene called RNA interference. Recently, RNA interference has been developed to interrupt the expression of target genes, especially to reduce the expression of problem genes.

RNA interference is characterised by several steps involving multiple interactions between RNA and protein. The initial step occurs in which dsRNA is cut into short small interfering RNAs (siRNA) which are 21-25 nucleotides in length (Buchon and Vaury, 2006) (Fig 1.3). Previously, the signal of interference was introduced as sense or anti-sense RNA. However, RNAi using an amplicon cassette or self-complementary hairpin RNA is more effective to silence the gene of interest (Chuang and Meyerowitz**,** 2000).The degradation of dsRNA occurs in both directions involving the RNAase III family and more particularly an enzyme called dicer. Most animals possess a single dicer while in plants, such as *Arabidopsis* and rice, have four and six respectively (Watson *et al*., 2005). The dicer-siRNA complex incorporates the RNA-induced silencing complex (RISC) unwinding it to create single stranded siRNA. The single stranded siRNAs guides RISC to the complementary strand RNA as the target of degradation (Reynolds *et al*., 2004).

In the project we generate Arabidopsis thaliana lines with suppressed *Atnda1* utilizing RNA Interference.

METHOD

Plant material

Wild type *Arabidopsis thaliana* ecotype Columbia was the project. The seeds were sterilized in 5 µL of 0.09% Tween 20 (Polyoxyethylene-SorbitanMonolaurate) (SIGMA) was added into 10 mL of 4% Bleach (EXPKleen, SA).

Bioinformatics and sequence analysis

The sequences of all related gene were provided from The *Arabidopsis* Information Resource (TAIR, http://www.*Arabidopsis*.org/) and the Munich Information Centre for Protein Sequences (MIPS http://mips.helmholtz-muenchen.de/plant/athal/). The alignment of nucleotides was performed by the alignment program from Geneious (http://www.geneious.com/), using Clustal X (Thompson *et al*., 1997).

Agarose gel electrophoresis

Electrophoresis was performed in systems from BIO-RAD (USA) with the EPS-300 Electrophoresis (Pharmacia Biotech, Sweden) used as power supply. Agarose gels were visualised under ultraviolet light on a BIO-RAD Transilluminator and imaged using a Kodak Digital Camera.

Bacteria and vectors

Bacteria *E. coli* DH5α was chosen for transformation works with plasmid pGEM carrying approximately 200 bp of *nda1* gene. For the purpose of cloning, a specific sequence of *nda1* using Gateway® Technology, pDON™221 was used as donor vector in BP reaction to create an entry vector and pAGRIKOLA utilised as destination vector in LR reaction. The destination vector was transformed into *Agrobacterium* to infect the targeted plants.

Preparation of competent *E. coli* DH5α and transformation

The culture was put on a shaker set at 180 rpm in at 37 ^{0}C and the absorbance at 600 nm of the culture was adjusted not more than 0.5. Two aliquots were provided with 50 mL each and centrifuged at 4000 g and 4 ^{0}C for 10 minutes in a refrigerated centrifuge 3-16PK from SIGMA. The supernatant was discarded and the cells were resuspended in 10 mL cold 0.1 M CaCl2. The resuspended cells were spun again at 4000 g, 4 ^{0}C for 10 minutes. Transformation was done by heat shock to transfer pGEM-*nda1* into competent *E. coli* DH5α.

PCR screening of the presence of pGEM-*nda1* in transformed colonies

The presence of pGEM-*nda1* in the colonies was confirmed by PCR using the primers which were NDA1TF 5' TCCACATTTGGAATTAATGGC 3' and NDA1Trev 5' CTCTCTCAGCCACCTTCATTG 3' .Three colonies were chosen and one colony of *E. coli* DH5α was used as sample for negative control. The amplification reaction used primer specific for *nda1*.

Plasmid extraction of transformed *E. coli* DH5α

The plasmid was extracted from the culture and purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA).

Sequencing of pGEM carrying *nda1* gene

Two reactions were performed in separate tubes and followed the instruction and recommendation of Australian Genome Research Facility (AGRF).

Designing gateway cloning primers

Gateway cloning primers for nda1 were designed for the next steps.

RNA extraction of wild type plant and Reverse transcription

RNA extraction was done using RNAeasy® Plant Mini Kits (Qiagen, USA) and following the manufacturer's instructions and protocols.Reverse transcription reaction was conducted using iScript™ cDNA Synthesis Kit (BIO-RAD Laboratories, California) and following manufacturer's instruction and protocols.

Amplification of cDNA using Phusion® High-Fidelity DNA Polymerase.

The PCR reaction was prepared as the recommendation in guidelines for using Phusion® DNA polymerase. The purified *attB*- PCR products were then preceded to BP recombination reaction.

Performing BP recombination reaction and transforming competent cells

BP recombination reaction was performed using a BP Recombination Reaction Kit (Invitrogen, California) and following the manufacturer's protocols and instructions.

Sequencing entry clones

Sequencing was done to ensure the correct sequence of entry clones derived from BP recombination reaction with pDONR 221. The PCR was performed by following the instructions and recommendations from Australian Genome Research Facility (AGRF) using M13 sequencing primers (forward and reverse).

Growing *E. coli* carrying pAGRIKOLA as the destination vector.

An overnight plate containing *E. coli* carrying pAGRIKOLA has been provided from the previous project. The plasmid was purified using plasmids preps (Promega, USA). The size of the plasmid was confirmed by gel electrophoresis and the concentration and the purity of purified plasmid DNA was quantified by Nano-drop.

Performing LR recombination reaction and transforming competent cells

The LR reaction has similar steps to the BP reaction but uses different components. This reaction was also performed using the LR combination Reaction Kit (Invitrogen, California) and following the manufacturer's protocols and instructions. The presence of pAGRIKOLA-RNAi-*nda1* in the colonies was confirmed by PCR. The products were run in Agarose Gel Electrophoresis.

Producing competent cells of *Agrobacterium tumefaciens* and transformation.

The competent *Agrobacterium* was produced by growing them selective LB and put on a shaking incubator at 28 0 C. Centrifugation using GSA Sorval rotor of centrifuge at 7000 rpm, 4 ^{0}C for 20 minutes.The pellets in 10 % glycerol (40 µL) were transformed with 2-4 ng (1 µL) of pAgrikola-RNAi-*nda1* using the Gene pulser II system (Bio-Rad, USA).

Floral dipping

The culture of the Agrobacterium was spun using GSA rotor of Sorvall centrifuge at 6000 rpm, 4^{0}C for 10 minutes. The pellets of Agrobacterium were resuspended in 5% sucrose until the optical density of the solution was at 600 nm between 0.6 and 1. The Silweet 1-77 was added (0.05 % v/v) into the solution to resuspend the Agro pellets. The mixture was then poured into a container. The dipping was done by inverting the pots and agitating the flowers into the mixture for 30 seconds. The plants were covered with plastic wrap to maintain high humidity during the first 12-24 hour after inoculation. The pots were then covered with a black cloth to create low light or dark conditions. The plants were left until the siliques were brown and dry.

Seed collection

The seeds were harvested by cutting the seed shoots using scissors and put into a paper bag. The seed shoots were dried at room temperature for several days. The seeds were stored in an Eppendorf at room temperature.

Screening of the putative transformants

Seeds were sterilized and plated onto selective MS media .The wild type was plated as negative control and the seeds containing pAGRIKOLA were the positive control. The plates were then placed

into a growth chamber at 20 ^{0}C with 16 hours of light and 8 hours of dark. The survived plants from selective plates were observed for one or two weeks of growth.

RESULT AND DISCUSSION

According to the unique sequence of *nda1*, the primers were designed by following the instruction of Gateway® Technology (Invitrogen ™) user manual. To generate the PCR products attached with *attB* sites, the forward and reverse primers must contain four guanine (G) residues at the 5' ends followed by 25 base pairs *attB1* site specifically for forward primer and 25 base pairs *attB2* for reverse primer. The primers were then used in amplification of *nda1* to create RNAi-*nda1* cassette with the expected size 150 base pairs *attB1 Forward primer* 5'-GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC- TTCCACTTCGCCACGT- 3' *attB2 Reverse primer* 5'-GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GT-GATTCAATTTCAAAGTCAATA-3'

Generation and amplification *Atnda1*

The competent cells were resuspended in cold CaCl2 proceeding to transformation by adding purified plasmid pGEM carrying approximately 180 bp *Atnda1* by heat shock. PCR was performed and the products The products visualised in agarose gel showed that all colonies contained the correct size of the insertion (Fig 1).

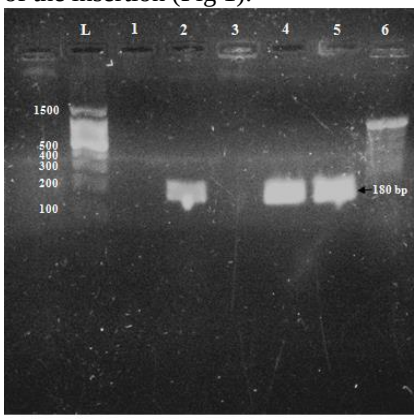

Figure 1. PCR screening of the presence pGEM-*nda1* in *E. coli* DH5α. The products were run in 2 % Agarose gel showing that three of four colonies (lane 2, 4 and 5) carried the transformed *nda1* gene with 180 bp length.

Creation of *nda1*-RNAi cassette utilising the Gateway cloning.

The Gateway Cloning system possesses two steps of recombination reactions, BP and LR reactions. In BP reaction, the *attB- nda1* was attached to the donor vector (pDONR 221) from Invitrogen using BP clonase. Reaction using another vector pEXp7-tet was decided to be the positive control. The recombinants were transferred into One Shot® OmniMAX 2-T1® chemically competent *E. coli* by heat shock and the transformed *E. coli* was spread onto selective plates. PCR screening using Gateway primers showed that the colonies carried the correct size of the insertion (Fig 2). It was expected that the sequence of entry clones was similar with extracted *nda1*. As expected, the results of the alignments showed that those sequences were similar to extracted *nda1* with the percentage of pairwise identity were 99.3 % and 100.0 % (reversed sequence) (Figure not shown). The LR reaction was performed to combine the purified entry clones carrying *attL1-nda1-attL2* to *attR*1-ccdB-*attR*2 in pAGRIKOLA. This vector is designed to express sense and anti-sense of the *nda1*-RNAi. PCR screening showed that the transformed colonies had the correct size of insertions (Fig.3). The expression clones were then purified for use in the subsequent step.

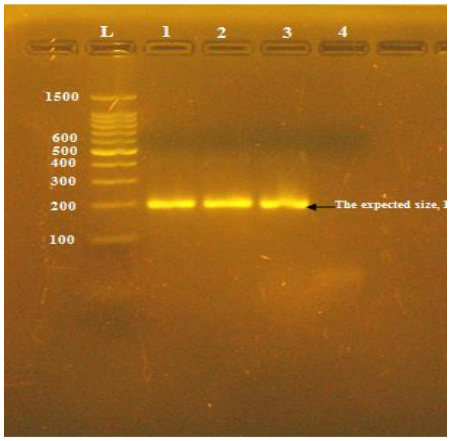

Figure 2. PCR screening of colonies carrying pDONR-*nda1*. The visualised products in 2 % agarose gel showed that the colonies carried the correct DNA plasmid (lane 1, 2, and 3). L = 100 bp DNA marked and 4 = negative control (water).

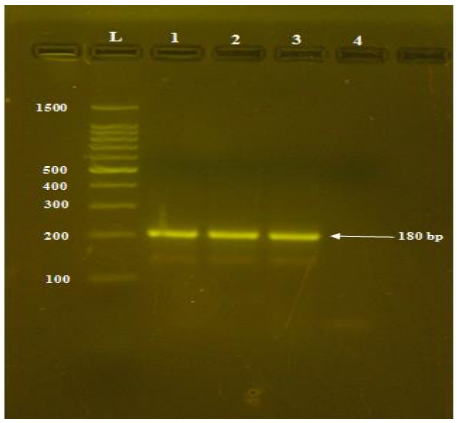

Figure 3. PCR screening of the presence of pAGRIKOLA-*nda1*-RNAi in *E. coli* colonies. The products were run in 2 % agarose gel showing that all colonies carried the correct size of the insertion (lane 1, 2, and 3). The marker was a 100 bp DNA ladder. Lower bands were primer dimers.

Confirmation of the putative transformants

Selection of the transformants has been done on selective plates. Because pAGRIKOLA carries a BASTA resistant gene (*Bar* gene), antibiotic was added into MS media. Another antibiotic, cefotaxime, also was used to avoid *Agrobacterium* contamination. Two controls were included to ensure the screening; one was wild type seeds as negative control and another was the seed carrying pAGRIKOLA as positive control. The seeds were planted onto MS media containing both antibiotics. Contamination of *Agrobacterium*, other bacteria and even fungus is the main issue in this screening. Another issue is that wild type can still grow in the selective plates. The effort to screen the seed has been repeated by preparing new stock for cefotaxime and using new sterilised seeds. However, contamination still occurred and the wild type still grew. It was decided to apply BASTA with different concentrations; 5 mg/ml and 10 mg/ml. It was observed that all the seeds suffered and bleached in plates containing5 mg/ml and 10 mg/ml of BASTA (Fig 4) and contamination still occurred at the one week germination stage. It can be concluded that the screening must be repeated.

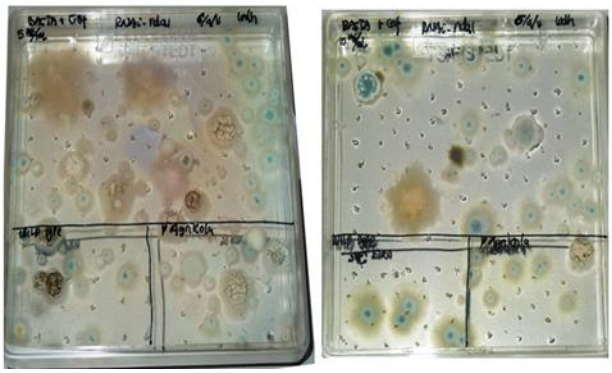

Figure 4. screening of the putative transformants.

The plates contain different concentrations of BASTA, 5 mg/ml and 10 mg/ml with combination of cefotaxime 200 mg/L. The seeds suffered and bleached at the one week germination stage in both plates. However, contamination still occurred. Therefore, the observation was done at further stages of growth.

DISCUSSION

Generation of mutant *Arabidopsis* with suppressed *Atnda1* by RNA interference

Many studies have been conducted for type II NAD(P)H dehydrogenases. However, their roles and functions are still unclear, especially *Atnda1*. RNA interference has been advanced for use to disrupt the expression of genes in organisms leading to the determination of its functions (Krysan, *et al*., 1999).

There is no doubt that double stranded RNA exhibits a potent and specific interference against any of variety of targeted genes (Montgomery and Fire, 1998). Therefore, double stranded *nda1*-RNAi silencing was chosen for this project, rather than sense or anti sense one. To express the RNA silencing, pAGRIKOLA has been nominated as it contains several advantageous features for Gateway technology (Wesley *et al*., 2001). The presence of two antibiotic resistance genes; *bar* gene (BASTA) and NPTII (kanamycin), offers convenient screening for the transformed plants (Hilson *et al*., 2004).

Arabidopsis floral dip transformation has useful features for transferring the cassette. Besides being simple to perform, it offers a high rate of success in which 1 % of the progeny seedlings are transformants (Zhang *et al*., 2006). For this purpose, the purified cassette was transformed into the *Agrobacterium tumefaciens* strain GV3101 and grown in selective broth before the dipping. Wild type *Arabidopsis* has grown healthily and vigorously as it is a key point of successful plant inoculation that in turn will produce plentiful seeds (Bent, 2006).

The screening of putative transformants was performed by germinating the seeds in selective media. The media contains BASTA which allows only transgenic plants to grow. Cefotaxime is a cephalosporin antibiotic which has a broad spectrum of activity against gram-negative and gram-positive bacteria (Silva and Fukai, 2001). Therefore, to eliminate *Agrobacterium*, cefotaxime was used.
Contamination is the main problem in screening of the transgenic plants in this project. Fungal and bacterial contaminations have occurred during germination even though cefotaxime has been applied in growth media. This may be due to the resistance of the microorganisms to the amount of cefotaxime which had been added (200 mg/L). This is less than the 500 mg/L of cefotaxime as suggested by Nauerby et al (1997). However, sterile condition while preparing the media and seeds, and during planting must be fully observed and maintained to avoid any possible contamination. Another issue is the growth of unexpected plants. During the first effort at screening, wild type still germinated as the transgenic plants did. Therefore, we used different amount of BASTA; 5 mg/mL and 10 mg/mL. All the shoots of the wild type and transformants suffered and bleached one week after germination. Unfortunately, contamination still occurred and the screening was terminated due to the time constraints.

CONCLUSION

We cannot ensure that transgenic *Arabidopsis* with suppressed *Atnda1* has been successfully generated since the screening was not

completed. However, alignment of *nda1* sequence in pDON221 and the reference sequence of *Atnda1* (AT1g07180) has confirmed that the insertion was 100% similar.

REFERENCES

Bent, A (2006) *Arabidopsis thaliana Floral Dip Transformation Method*, from Method in Molecular Biology, Vol.343:87-104, edited by Kan Wang, Humana Press, Totowa, NJ.

Carrie, C., Murcha, M.W., Kuehn, K., Duncan, O., *Bar*thet, M., Smith, P.M., Eubel, H., Meyer, E., Day, D.A., Millar, H., and Whelan, J. (2008) *Type II NAD(P)H Dehydrogenases are Targeted to Mitochondria and Chloroplasts or Peroxisomes in Arabidopsis thaliana*, FEBS Letters, 582: 3073-3079.

Chuang, C.F. and Meyerowitz, E.M (2000) *Specific and Heritable Genetic Interference by Double-Stranded RNA in Arabidopsis thaliana*, Plant Biology, 97: 4985-4990.

Coffee, C.J. (1999) *Quick Look: Metabolism*, Hayes *Bar*ton Press, USA.

Finnegan, P.M., Soole, K.L., and Umbach, A.L (2004) *Alternative Mitochondrial Electron Transport Proteins in Higher Plants*, In Day, D.A, Millar, A.H and Whelan, J (Eds.) *Plant Mitochondria: From Genome to Function*, Kluwer Academic Publishers,

Hilson *et al.*(2004) *Versatile Gene-Specific Sequence Tags for Arabidopsis Functional Genomics: Transcript Profiling and Reverse Genetics Applications*, Genome Research, 14: 2176-2189.

Karp, G. (1996) *Cell and Molecular Biology; Concepts and Experiments*, John Wiley &Sons, Canada.

Krysan, P.J., Young, J.C., and Sussman, M.R. (1999) TDNA as an Insertional Mutagen in *Arabidopsis*, The Plant Cell, 11: 2283-2290.

Michalecka, A.M., Agius, S.C., Moller, I.M., and Rasmusson, A.G. (2004) *Identification of A Mitochondrial External NADPH dehydrogenase by Overexpression in Transgenic Nicotianasylvestris*, The Plant Journal, 37: 415-425.

Michalecka, A.M., Svensson, A.S., Johansson, F.I., Agius, S.C., Johanson, U., Brennicke, A., Binder, S., and Rasmusson, A.G. (2003) *Arabidopsis Genes Encoding Mitochondrial Type II NAD(P) H Dehydrogenases Have Different Evolutionary Origin and Show Distinct Response to Light*, Plant Physiology, 133: 642-652.

Nauerby, B., Billing, K., and Wyndaele, R. (1997) *Influence of The Antibiotic Timentin on Plant Regeneration Compared to Carbenicillin and Cefotaxime in Concentration Suitable for Elimination of Agrobacterium Tumefaciens*, online journal of Plant Science, 123:169-177, issued 2 march 1997.

Rasmusson, A.G and Agius, S.C (2001) Rotenone-insensitive NAD(P)H Dehydrogenases in Plants: Immunodetection and Distribution of Native Proteins in Mitochondria, Plant Physiol. Biochem, 39: 1057-1066.

Reynolds, A., Leake, D., Boesa, Q., Scaringe, S., Marshall, W.S., and Khvorova, A (2004) *Rational siRNA Design for RNA Interference*, Nature Biotechnology, 22: 326-330.

Smith, C.A. (2010) *Altered Expression of The Non-Phosphorylation Electron Transport Chain Effects Growth and Stress Tolerance in Arabidopsis thaliana*, Doctor of Philosophy, Flinders University.

Svensson, A.S. and Rasmusson, A.G. (2001) Light-dependent Gene Expression for Proteins in The respiratory Chain of Potato Leaves, The Plant Journal, 28: 73-82.

Taiz, L. and Zeiger, E. (2002) *Plant Physiology Third Edition*, Sinauer Associates, Massachusetts.

Vanlerberghe, G.C., and McIntosh, L. (1997) *Alternative Oxidase: From Gene to Function*.Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 703-734.

Watson, J.M., Fusaro, A.F., Wang, M., and Waterhouse, P.M. (2005)*Minireview: RNA Silencing Platforms in Plants*, FEBS Letters, 579: 5982-5987.

Wesley, S.V, Helliwell, C.A., Smith, N.A., Wang, M., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., and Stoutjesdijk, P.A. (2001) *Construct Design for Efficient, Effective and High-throughput Gene Silencing in Plants*, Plant J.,27:581-590.

Zhang, X., Henriques, R. Lin, S.S., Niu, Q.W., and Chua, N.H. (2006) *Agrobacterium-mediated Transformation of Arabidopsis thaliana Using The Floral Dip Method*, Nature Protocols, 1: 641-646.